

TABLE 1. *Production of nitric oxide and tumoricidal properties in mouse macrophages by liposomes containing MTP-PE, CGP31362 and JT3002*

Concentration of MLV (nmol/well)	MLV-HBSS		MLV-MTP-PE		MLV-31362		MLV-JT3002	
	NO (μ M)	Cytotoxicity (%)	NO (μ M)	Cytotoxicity (%)	NO (μ M)	Cytotoxicity (%)	NO (μ M)	Cytotoxicity (%)
50	8	4	4	19	28*	84*	30*	86*
25	5	0	2	14	26*	74*	29*	80*
12	1	1	2	10	23*	79*	23*	84*
6	1	2	2	5	22*	72*	22*	70*
3	1	0	2	4	20*	75*	22*	68*

Macrophages (1×10^5 /well) were incubated with the indicated concentrations of MLV in medium containing 10 U/ml IFN- γ . All MLV contained 1 mg immunomodulator/300 μ M phospholipids. NO production (nitrite/nitrate) was determined one day later. The cultures were washed and 1×10^5 [3 H]TdR-labeled A375P cells were added. Assays were terminated 72 h later. Macrophages incubated in medium alone (negative control) produced 0.2 μ M NO and 10% cytotoxicity. Macrophages in medium containing LPS (1 μ g/ml) and IFN- γ (10 U/ml) produced 26 μ M NO and 48% cytotoxicity ($P < 0.001$). The values are the mean of triplicate cultures. Variation from the mean did not exceed 10%. These are the results of one representative experiment of four.

* $P < 0.001$.

TABLE 2. *Minimal concentration of liposome-JT3002 required to induce production of nitric oxide in murine macrophages*

Lipid concentration (nmol/well)	NO (μ M)			
	JT3002 (0.1 mg)	JT3002 (0.02 mg)	JT3002 (0.004 mg)	JT3002 (0.0008 mg)
25	27 ^a	23 ^a	10 ^a	11
12.5	26 ^a	20 ^a	14 ^a	9
6.2	24 ^a	17 ^a	12 ^a	7
3.1	24 ^a	16 ^a	10	7
1.6	21 ^a	13 ^a	9	7
0.8	17 ^a	11	9	7
0.4	19 ^a	11	10	7
0.2	18 ^a	10	10	6

Macrophages (1×10^5 /well) were incubated in medium containing 10 U/ml IFN- γ (control) or medium containing 10 U/ml IFN- γ and different concentrations of liposomes containing 0.1 mg, 0.02 mg, 0.004 mg, or 0.008 mg JT3002 in 300 μ M phospholipids. NO production was determined 24 h later. The values are the mean NO production in μ M of triplicate cultures. Variation from the mean did not exceed 10%. Macrophages incubated with medium plus IFN- γ or medium containing IFN- γ plus LPS produced 9 and 25 μ M NO, respectively. This is one representative experiment of three.

^a $P < 0.001$.

TABLE 3. Activation of tumoricidal properties in macrophages from iNOS knockout mice

Lipid concentration (nmol/well)	NO (μ M)			Cytotoxicity (%)		
	+/+ mice	+/- mice	-/- mice	+/+ mice	+/- mice	-/- mice
50	21 ^a	14 ^b	0	93 ^a	91 ^a	7
25	20 ^a	14 ^b	0	93 ^a	89 ^a	1.5
10	17 ^a	12	0	85 ^a	62 ^a	0
5	16 ^a	11	0	31 ^a	51 ^a	0
0	0	0	0	0	0	0
LPS (1 μ g/ml)	20 ^a	13	0			

Macrophages (1×10^5 /well) were incubated in medium containing 10 U/ml IFN- γ (control) or medium containing 1 μ g/ml LPS (positive control), or medium containing different concentrations of MLV containing 0.1 mg JT3002/300 μ M phospholipid. After 20 h incubation, the cultures were washed and 1×10^4 [3 H]TdR-labeled K-1735 M2 (shown) or CT-26 (not shown) cells were added. NO production (μ M/10⁶ macrophages) was determined after 20 h and cytotoxicity was determined after 72 h. The values are the mean of triplicate samples. Variation from the mean did not exceed 15%. This is one representative experiment of three.

^a $P < 0.01$.

^b $P < 0.05$.

TABLE 4. *Activation of tumoricidal properties in macrophages from LPS-responsive (C3H/HeN) and -nonresponsive (C3H/HeJ) mice*

Lipid concentration (nmol/well)	C3H/HeN mice		C3H/HeJ mice	
	NO (μ M)	Cytotoxicity (%)	NO (μ M)	Cytotoxicity (%)
20	23 ^a	35 ^a	32 ^a	40 ^a
2	11	28 ^a	26 ^a	32 ^a
0.2	2	13	13	27 ^a
0.02	5	7	9	11
0	2	3	0	6
LPS (1 μ g/ml)	23 ^a	36 ^a	8	12

Macrophages (1×10^5 /well) were incubated in medium containing 10 U/ml IFN- γ (control), or medium containing 1 μ g/ml LPS (positive control), or medium containing different concentrations of MLV containing 0.1 mg JT3002/300 μ M phospholipid. After 20 h incubation, the cultures were washed and 1×10^4 [3 H]TdR-labeled K-1735 M2 cells were added. NO production (nitrite) was determined after 20 h and cytotoxicity was determined after 72 h. The values are the mean of triplicate samples. Variation from the mean did not exceed 10%. This is one representative experiment of three.

^a $P < 0.01$.

TABLE 5. *Duration of tumoricidal activity in macrophages incubated with liposomes containing JT3002*

Days post-activation	NO (μ M)		Cytotoxicity (%)	
	Medium	JT3002	Medium	JT3002
1	0.9	31.8 ^a	5.9	49.7 ^a
2	1.3	34.0 ^a	6.6	19.8 ^b
3	0.7	27.7 ^a	4.1	19.2 ^b
4	4.9	4.0	5.9	4.8
<u>Reactivation</u>				
5	2.2	33.7 ^a	3.0	41.0 ^a

Macrophages (1×10^5 /well) were incubated in medium containing 10 U/ml IFN- γ (control) or medium containing 10 U/ml IFN- γ plus 1 nmol/well of MLV containing 0.1 mg JT3002/300 μ M phospholipid. After 20 h incubation, the cultures were washed and fresh medium was added for 0, 1, 2, 3, or 4 days. At the different time points, 1×10^4 [³H]TdR-labeled CT-26 cells were added. NO production (nitrite/nitrate) was determined at the indicated times. Cytotoxicity was determined after 72 h of continuous tumor-cell-macrophage interaction. The values are the mean of triplicate cultures. Variation from the mean did not exceed 10%. This is one representative experiment of two.

^a $P < 0.001$.

^b $P < 0.01$.

TABLE 7
Combination Therapy of MTP-PE and CPT-11 for Mouse
CT-26 Colon Cancer Liver Metastasis

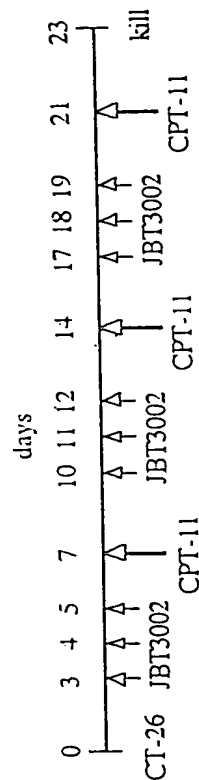
Oral treatment	CPT-11	Spleen		Liver	
		Weight (g)	Tumor size (mm)	Weight	Median no. metastases
Saline	Saline	1.5 ± 0.1	1.4 ± 0.7	7.4 ± 1.6	>100
Saline	50 mg/kg	0.6 ± 0.2	8.3 ± 2.0	2.0 ± 0.3	30
Saline	100 mg/kg	-----All mice died.-----			
MTP-PE	50 mg/kg	0.6 ± 0.2	10.4 ± 2	2.2 ± 0.7	30
MTP-PE	100 mg/kg	0.3 ± 0.1	5.6 ± 2	1.2 ± 0.1	4

Table 10. Therapy of experimental liver metastasis produced by murine CT-26 colon carcinoma with CPT-11 in combination with either MLV-JBT 3002 or free-form (FF) JBT 3002

Treatment	Spleen (primary)			Liver (metastasis)		
	ΔBW^a (%)	Incidence	Tumor volume (mm ³)	Incidence	Median (range)	Liver weight (g)
MLV-HBSS	6.4	5/5	567 ± 94	5/5	46, 56, 72, >100, >100	3.5 ± 1.6
MLV-HBSS + CPT-11	-1.7	5/5	140 ± 30 ^c	5/5	12, 15, 18, 39, 73	1.8 ± 0.3 ^b
MLV-JBT3002 (1.0 µg/dose) + CPT-11	-0.4	5/5	56 ± 29 ^c	2/5	0, 0, 0, 6, 12	1.6 ± 0.2 ^b
MLV-JBT3002 (0.1 µg/dose) + CPT-11	-0.8	5/5	72 ± 15 ^c	3/5	0, 0, 4, 8, 79	1.6 ± 0.2 ^b
FF-JBT3002 (1.0 µg/dose) + CPT-11	-3.9	5/5	202 ± 69 ^b	5/5	7, 25, 37, 53, 81	1.8 ± 0.4 ^b
FF-JBT3002 (0.1 µg/dose) + CPT-11	0	5/5	85 ± 23 ^c	3/5	0, 0, 9, 13, 35	1.5 ± 0.3 ^b

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Five BALB/c mice per group were given intrasplenic injection of 1×10^4 CT-26 cells on day 0. Mice were treated with repeated oral feedings of MLV-JBT3002 (at either 1.0 or 0.1 µg/dose, 5 µmol PCPS MLV), or FF-JBT3002 (at either 1.0 or 0.1 µg/dose) thrice weekly for 3 weeks beginning 3 days after tumor cell inoculation, in combination with 100 mg/kg CPT-11 i.p. once a week (on day 7, 14, and 21). All groups were killed on day 23.



^aThe rate of body weight reduction was calculated with the formula: $\Delta BW (\%) = (A - B - 1) \times 100$, where A = mean body weights of mice at death, and B = mean body weights of mice on day 0.

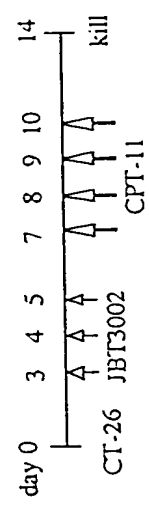
^b $P < 0.05$, ^c $P < 0.005$, compared with MLV-HBSS

Table 12. Therapy of experimental liver metastasis produced by murine C1-26 colon carcinoma with MLV-JBT 3002 or free-form (FF) JBT 3002 at different doses in combination with either MLV-JBT 3002 or free-form (FF) JBT 3002 at different doses

Treatment	Spleen (primary)		Liver (metastasis)			
	ΔBW^a (%)	Incidence	Tumor volume (mm ³)	Incidence no.	Liver weight (g)	
MLV-HBSS + saline	5.1	5/5	153 ± 62	5/5	23, 26, 71, >100, >100	2.4 ± 1.0
MLV-HBSS + CPT-11	-17.6	5/5	52 ± 30	2/5	0, 0, 0, 1, 6	1.2 ± 0.1
MLV-JBT3002 (1.0μg/dose) + CPT-11	-1.5	5/5	45 ± 10	0/5	all 0	1.4 ± 0.1
FF-JBT3002 (1.0μg/dose) + CPT-11	-2.4	5/5	48 ± 8	2/5	0, 0, 0, 3, 5	1.4 ± 0.03
FF-JBT3002 (0.1μg/dose) + CPT-11	-2.2	5/5	50 ± 16	1/5	0, 0, 0, 0, 3	1.4 ± 0.2
FF-JBT3002 (0.01μg/dose) + CPT-11	0.4	5/5	29 ± 26	4/5	0, 2, 2, 26, 27	1.6 ± 0.1
FF-JBT3002 (0.001μg/dose) + CPT-11	-6.9	5/5	56 ± 25	1/5	0, 0, 0, 0, 3	1.4 ± 0.2
FF-JBT3002 (0.0001μg/dose) + CPT-11	-15.4	5/5	28 ± 20	3/5	0, 0, 1, 2, 5	1.1 ± 0.1

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BALB/c mice were injected into the spleen with 1×10^4 viable CT-26 cells on day 0. Mice were treated with oral feedings of 5 µmol MLV-HBSS, MLV-JBT3002 (1 µg/dose), or FF-JBT3002 (at either 1.0, 0.1, 0.001, or 0.0001 µg/dose) for 3 consecutive days beginning 3 days after tumor cell inoculation. Seven days later, groups of mice received 4 daily i.p. injections of 100 mg/kg CPT-11. All groups were killed on day 14.



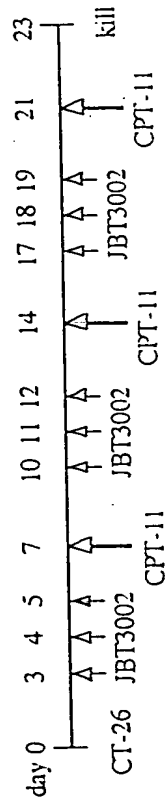
^aChanges in body weight were calculated by the formula: $\Delta BW\ (\%) = \frac{(A - B)}{B} \times 100$, where A = mean body weight of mice at death, and B = mean body weight of mice on day 0.

Table 13. Therapy of experimental liver metastasis produced by murine CT-26 colon carcinomas with once weekly CPT-11 injections in combination with either MLV-JBT 3002 or free-form (FF) JBT 3002 at different doses

Treatment	Spleen (primary)			Liver (metastasis)	
	ΔBW^a (%)	Incidence	Tumor volume (mm ³)	Incidence	Liver weight (g)
MLV-HBSS + saline	3.1	5/5	699 ± 322	5/5	89, >100, >100, >100
MLV-HBSS + CPT-11	1.2	5/5	334 ± 88	5/5	42, 42, 45, 56, 79
MLV-JBT3002 (1.0µg/dose) + CPT-11	1.3	5/5	157 ± 96	4/5	0, 1, 9, 11, 13
FF-JBT3002 (1.0µg/dose) + CPT-11	-1.4	5/5	235 ± 78	5/5	34, 41, 56, 70, 88
FF-JBT3002 (0.1µg/dose) + CPT-11	-0.2	5/5	189 ± 13	5/5	3, 12, 16, 24, 34
FF-JBT3002 (0.01µg/dose) + CPT-11	0.3	5/5	214 ± 45	5/5	2, 4, 13, 31, 40
FF-JBT3002 (0.001µg/dose) + CPT-11	2.5	5/5	237 ± 20	5/5	31, 42, 47, 58, 69
FF-JBT3002 (0.0001µg/dose) + CPT-11	2.3	5/5	225 ± 34	5/5	30, 32, 48, 52, 83

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BALB/c mice were injected into the spleen with 1×10^4 viable CT-26 cells on day 0. Groups of mice were treated with oral feedings of 5 µmol MLV-HBSS, MLV-JBT3002 (1 µg/dose), or FF-JBT3002 (at either 1.0, 0.1, 0.001, or 0.0001 µg/dose) thrice weekly for 3 weeks beginning 3 days after tumor cell inoculation. Some mice received an i.p. injection of 100 mg/kg CPT-11 once a week (on days 7, 14, and 21). All groups were killed on day 23.



^aChanges in body weight were calculated by the formula: $\Delta BW (\%) = (A - B) / B \times 100$, where A = mean body weight of mice at death, and B = mean body weight of mice on day 0.

Table 14. Therapy of experimental liver metastasis produced by murine CT-26 colon carcinomas with intensive CPT-11 injections in combination with either MLV-JBT 3002 or free-form (FF) JBT 3002 at different doses

Treatment	Liver metastasis		Spleen tumor		Liver weight (g)
	$\Delta BV_{1,4}$ (%)	ΔBV_{21} ^a (%)	Incidence	Mean tumor volume (mm ³)	
Control	2.9	6.9	5/5	353 ± 29	3.4 ± 1.1
CPT-11	-24.0	ND	5/5 ^b	35 ± 16	1.2 ± 0.2
MLV-JBT 3002 (1.0 µg/dose) + CPT-11	-9.4	-7.6	5/5	75 ± 64	1.5 ± 0.1
FF-JBT 3002 (0.05 µg/dose) + CPT-11	-6.8	-6.0	5/5	83 ± 70	1.7 ± 0.0

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BALB/c mice were injected into the spleen with 1×10^4 viable CT-26 cells on day 0. Mice were treated with oral feedings of 5 μ mol MLV-JBT 3002 (1 μ g/dose), or FF-JBT 3002 (0.05 μ g/dose) for 3 consecutive days beginning 3 days after tumor cell inoculation. Seven days later, groups of mice received 4 daily i.p. injections of 100 mg/kg CPT-11. All groups were killed on day 21.



Changes in body weight were calculated by the formula: $\Delta BW (\%) = (A - B) / B \times 100$, where A = mean body weight of mice on the indicated day, and B

= mean body weight of mice on day 0.

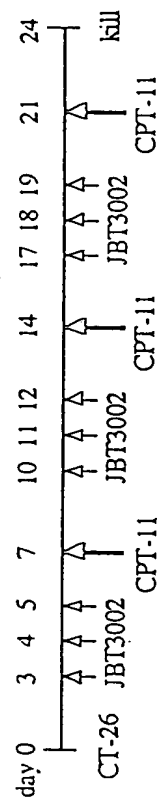
^b All mice died during therapy (3 mice on day 15 and 2 mice on day 16).

ND, not determined.

Table 4. Therapy of experimental liver metastasis produced by murine CT-26 colon carcinoma with once weekly CPT-11 injections in combination with oral JBT 3002

Treatment	Spleen tumor		Liver metastasis		P^a	Liver weight (g)
	Incidence	Mean tumor volume (mm ³)	Incidence	No.		
Control	10/10	574 ± 101	10/10	72, >100, >100, >100, >100 >100, >100, >100, >100, >100		4.3 ± 1.0
CPT-11	7/10	116 ± 32 ^b	8/10	0, 0, 1, 5, 6, 13, 33, 81, 85, >100	0.0005	2.0 ± 0.9 ^c
JBT 3002	8/10	241 ± 84	9/10	1, 2, 50, >100, >100, >100 >100, >100, >100, >100, >100		4.2 ± 1.6
JBT 3002 + CPT-11	6/10	76 ± 34 ^b	5/10	0, 0, 0, 0, 0, 1, 6, 7, 37, 57	<0.0001	1.7 ± 0.4 ^c

BALB c mice were injected into the spleen with 1×10^4 viable CT-26 cells on day 0. Groups of mice were treated with oral feedings of JBT3002 (0.05 µg dose) thrice weekly for 3 weeks beginning 3 days after tumor cell inoculation. Some mice received an i.p. injection of 100 mg/kg CPT-11 once a week (on days 7, 14, and 21). All groups were killed on day 24.



^aAs compared with control.

^b $P < 0.05$ as compared with control. ^c $P < 0.0001$ as compared with control.

Table 17. Induction of NO production in macrophages by free-form, formula 1, and formula 2 JBT 3002

1. Macrophages: TG-Mø from C57BL/6 mice.
2. Treatment of macrophages: Macrophages in 96-well plates (10^5 /well) were incubated for 24 hr with JBT in the presence or absence of IFN- γ (10 U/ml). Nitrite in the culture medium was then determined.
3. Results:

JBT conc. (ng/ml)	Free JBT		Formula-1 JBT (pH 1.5-7)		Formula 2-JBT (pH 8)	
	medium	IFN-g	medium	IFN-g	medium	IFN-g
10	8.4	60.9*	2	50.7	2	47.4
2	0	53.1	0	38.6	0	38.1
0.4	0	44.7	0	34.8	0	33.5
0.08	0	41	0	25.5	0	20
0.016	0	33.7	0	6.3	0	1.9
0.003	0	17.5	0	0.4	0	0.7
0.0006	n.d.	n.d.	0	0.5	0	2
0	0	0.6				

- nitrite: μ M.

LAL endotoxin test:

No endotoxin was detected in the free form JBT3002, Formula 1-JBT, and Formula 2-JBT at a concentration of 0.08 ng/ml of the reagent.

Table 8. Induction of NO production by JBT 3002.

1. Materials and Methods

- 1) Macrophages: C57BL/6 mice, TG-M ϕ , 10⁵ cells/well in 96-well plate.
- 2) Treatment: with 10 U/ml of IFN- γ and various concentrations of JBT3002 for 24 hr in 200 μ l/well MEM-5% FBS. Nitrite (100 μ l/well) was measured.

2. Results

\leftarrow TABLETS \rightarrow

JBT3002 (ng/ml)	Free form		filtered		unfiltered	
	Medium	IFN- γ	medium	IFN- γ	medium	IFN- γ
10	0.5	47.1	0	41.0	7.0	53.0
1	0	37.7	0	29.3	0	44.5
0.1	0	27.7	0	20.9	0	34.1
0.01	0	19.5	0	7.7	0	26.2
0.001	0	8.5	0	0	0	4.3
0.0001	0	0	0	0	n.d.	n.d.
0	0	0				

3. Endotoxin Test:

Endotoxin was not detected by the LAL assay in all of the three preparations of JBT3002 at concentration of 0.1 ng/ml.

4. CONCLUSION:

The contents in the tablet formulation did not alter the activity of JBT3002 in activation of macrophages in vitro.

Treatment start with CPT11: 7 days after orthotopic tumor cell injection

Treatment start with JBT3002: 3 days after orthotopic tumor cell injection

Treatment schedule:	wed	thurs	fri	sat	sun	mon	tues
JBT3002	JBT3002	JBT3002	JBT3002	-	-	CPT11	-

(animals were sacrificed 31 days after tumor cell injection)

CPT11		CPT11 + JBT 3002	
animal	Tumor weight (mg)	Incidence	Incidence
		liver met	liver met
		LN met	LN met
		WT/PC	WT/PC
1	80	-	-
2	375	-	-
3	241	-	-
4	0	-	-
5	98	-	-
6	0	-	-
7	318	-	-
8	137	-	-
9	205	-	-
10	67	-	-
Median	117.5	0/10	0/10
Max	375	7/10	1/10
Min	0		
Average	152.10		
St.Dev.	106.12		

Table 19B. Tumor weight and incidence of metastases of L3.6pl human pancreatic tumors in nude mice after 4 weeks treatment with 100 mg/kg CPT-11 i.p. once a week +/- oral feeding of JBT 3002 (tablet) 0.05 mcg/dose

Treatment start with CPT11: 7 days after orthotopic tumor cell injection
 Treatment start with JBT3002: 3 days after orthotopic tumor cell injection
 Treatment schedule: wed thurs fri sat sun mon tues
 JBT3002 JBT3002 JBT3002 - CPT11 -
 (animals were sacrificed 31 days after tumor cell injection)

Control (HBSS)		JBT - 3002		Incidence		LN met		liver met		WT/PC	
animal	Tumor weight (mg)	Incidence	LN met	WT/PC	Tumor weight (mg)	Incidence	LN met	liver met	WT/PC	LN met	WT/PC
1	534	-	++	-	862	-	++	-	WT	++	WT
2	556	-	++	WT/PC	871	-	+	-	-	+	-
3	483	-	++	-	981	+	++	(5)	WT	++	WT
4	831	+	++	-	621	-	++	-	WT	++	WT
5	955	+	+	-	362	-	+	-	-	+	-
6	73	+	++	-	733	-	++	-	-	++	-
7	578	+	++	-	559	-	++	-	-	-	-
8	723	++	++	-	820	+	++	(1)	-	+	-
9	701	-	++	WT	547	-	++	-	-	-	-
10		-	++	WT		-	++	-	-	-	-
Median	578	4/10	10/10	3/10	733	2/9	7/10				3/10
Max	955				981						
Min	73				362						
Average	603.78				706.22						
St.Dev.	176.64				163.53						

Table 19C. Tumor weight and incidence of metastases of L3.6pl human pancreatic tumors in nude mice after 4 weeks treatment with 100 mg/kg CPT-11 i.p. once a week +/- oral feeding of JBT 3002 (tablet) 0.05 mdg/dose

Treatment start with CPT11: 7 days after orthotopic tumor cell injection
 Treatment start with JBT3002: 3 days after orthotopic tumor cell injection
 Treatment schedule: wed thurs fri sat sun mon tues
 JBT3002 JBT3002 JBT3002 - - CPT11 -
 (animals were sacrificed 31 days after tumor cell injection)

therapy	tumor weight in mg median (range)	Incidence liver met.	LN met.
Control (HBSS)	578 (73 - 955)	4/10	10/10
JBT3002	733 (362 - 981)	2/9	7/10
CPT11	117.5 (0 - 375)	0/10	7/10
CPT11+JBT3002	157.5 (0 - 365)	0/10	1/10

oral JBT 3002 in nude mice

<u>Intensive</u>		7/20	7/27	
	M		M T W R F S S M T W R F S S	
#5594	T			
#5595				
#5596	T		C C C C	
#5597				
#5598	T		J J J	
#5599				
#5600	T		J J J C C C C	
#5601				
<u>Once a week</u>				
	M		M T W R F S S M T W R F S S	
#5602	T			
#5603				
#5604	T		C (7f)	C 5604: 75
#5605				5605: 50
#5606	T		J J J	J J J
#5607				
#5608	T		J J J C (7f)	J J J C
#5609				5608: 7f
				5609: 50

T: KM12sm 1x10⁶ i.spl
 J: FF-JBT3002 (0.05mcg/dose) oral
 C: CPT-11 (50mg/kg) i.p.

Table 2/. Therapy of experimental liver metastases produced by CT-26 murine colon carcinoma with CPT-11 i.p. plus oral

JBT 3002 (free-form or tablet) in BALB/c mice

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